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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Unrepaired or incorrectly repaired double strand breaks (DSBs) produced by ionizing radiation, exogenous DNA damaging chemicals, free oxygen radicals or programmed cellular mechanisms, can lead to deletions or mutations in the coding sequence of a gene, translocations, inversions, chromosome loss during or cell death. Accurate repair of DSBs is performed by a group of highly conserved genes termed the *RAD54* epistatis group consisting of *RAD51, RAD52, RAD54, RAD55, RAD57, RAD59,* and *RDH54/TID1*. A key intermediate in recombinational repair involves single-strands of the broken DNA invading and displacing DNA from an intact homologous duplex joint forming a heteroduplex DNA joint to be utilized as a template to replace lost or damaged DNA. In mammals, human genes central to homologous recombination interact with and their biochemical efficiency is governed by the tumor suppressors BRCA1 and BRCA2 indicating a role of recombinational repair in the suppression of cancer formation. The human Rad51 recombinase protein DNA pairing and strand exchange yielding heteroduplex DNA joints between ssDNA and dsDNA. Our biochemical studies address the manner in which hRad54 and hRad51 functionally interact to promote hRad51-mediated D-loop formation and hRad54 catalyzed DNA supercoiling and transient DNA strand opening.

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Table of Contents

INTRODUCTION

Maintaining the integrity of the genome is of paramount importance for cell viability. Therefore, proper repair of DNA damage is an essential component of the cellular process. One potentially lethal type of DNA damage are double-strand breaks (DSBs) that occur due to cellular exposure to ionizing radiation (IR), exogenous DNA damaging chemicals such as alkylating agents, and by endogenously produced free oxygen radicals. Unrepaired or incorrectly repaired DSBs can lead to deletions or mutations in the coding sequence of a gene, translocations, inversions, aberrant recombinational events during S-phase, chromosome loss during mitosis or cell death. Unlike single-strand DNA damage which can be repaired using information on the remaining undamaged DNA strand, double-strand breaks must either be repaired by joining and ligating the broken ends or by utilizing a homologous chromosome as a template to copy the lost or damaged DNA. For the accurate repair of DSBs, a repair mechanism referred to as homologous recombination (HR) or homology-directed repair (HDR), utilizes single-strands of the broken DNA to invade and displace a DNA strand from an intact homologous duplex joint that is then used as a template to replace lost or damaged DNA. In eukaryotic organisms homologous recombination is mediated by genes required for meiotic and mitotic recombination and for DSB repair collectively known as the *RAD52* epistasis group, consisting of *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1, MRE11* and *XRS2*. Extensive studies have shown that the encoded proteins of these genes are conserved among eukaryotes from the baker's yeast *Saccharomyces cerevisiae* to humans in both primary sequence and biochemical functions (reviewed by Paques and Haber, 1999; Sung et al, 2000). In humans, the importance in the *RAD52* epistasis group in DSB repair, the maintenance of genomic stability, and the suppression of breast and other cancers has been manifest by several studies indicating that the encoded products of key members of *RAD52* group genes interact with and are governed by the tumor suppressors BRCA1 and BRCA2 (reviewed in Dasika et al, 1999; Pierce et al, 2001). Notably, in response to DNA damage BRCA1 is hyperphosphorylated and effects the localization of the DNA end processing complex Rad50, Mre11 and Xrs2 to the ends of the DSBs. Genetic analysis indicated that BRCA1 is required for normal levels of homologous recombination and the repair of DSBs (Moynahan et al, 1999; reviewed in Pierce et al, 2001). Additionally, the human recombinase hRad51 interacts with BRCA2 though eight BRC repeats (Chen and al, 1998; Wong et al, 1997) and BRCA2 mutant cells are not only sensitive to DSB inducing agents but also show a marked reduction in recombination and DSB repair (Moynahan et al, 2001).

BODY

A myriad of genetic analysis and biochemical studies on these *RAD52* epistasis genes and their encoded products have provided insights indicating the temporal order of events in homologous recombination (reviewed in Paques and Haber, 1999; Sung et al, 2000). Consequently, members of the *RAD52* group genes have been divided into two categories. The first category includes members Rad50, Mre11, and Xrs2 function in the initial DNA end-processing of the double-strand break in the formation of 3' overhanging DNA while the second category members Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, and Rdh54 nucleate onto the ssDNA tails generated from the break processing reaction and then mediate the

promoting the key intermediate heteroduplex formation or D-loop with intact homologous duplex, either homologous chromosomes or heterochromatid (Petes et al, 1991; Shinohara and Ogawa, 1995).

hRad54 physically interacts with hRad51 - Human Rad51 (hRad51), structural and functional homolog to the yeast Rad51 and *E. coli* RecA DNA recombinases, is central in mediating DNA pairing and strand exchange. Like its homolog counterparts, hRad51 forms a highly extended right-handed nucleoprotein filament on ssDNA (reviewed in Yu et al, 2001). Once formed, the hRad51-ssDNA nucleoprotein filament has the ability to perform a search for a homologous duplex, mediate strand invasion, and catalyze the formation of DNA joints by a process termed homologous pairing and strand exchange (Bauman et al, 1996; Gupta et al, 1997; Sigurdsson et al, 2001).

Although *in vitro* and yeast two-hybrid system analyses indicate that hRad54, a member of the Swi2/Ssnf2 protein family, can bind hRad51 via its amino-terminus (Golub et al, 1997), association between mRad51 and mRad54 in mouse ES cells has been shown to require a prior treatment of the cells with a DNA damaging agent (Tan et al, 1999). We wished to examine whether purified hRad54 physically interacts with hRad51. To do so we coupled hRad51 to Affi-gel beads to be used as affinity matrix for binding hRad54. As shown in Figure 1, purified hRad54 was retained on Affi-hRad51 beads but not on Affi-beads that contained bovine serum albumin. Furthermore, when we used a less purified hRad54 fraction (~25% hRad54), hRad54, but not the contaminating protein species, bound to the Affi-hRad51 beads. The results thus indicate a direct and specific interaction between hRad51 and hRad54.

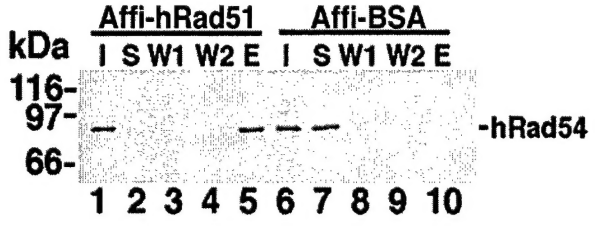


Figure 1. hRad54 interacts with hRad51. Purified hRad54 (1.2 μg) was mixed with Affi-beads containing either BSA (Affi-BSA) or hRad51 (Affi-Rad51) in 30 μl, washed twice with 50 μl buffer, followed by treatment of the beads with 30 μl SDS to elute bound hRad54. The starting material (I), supernatant (S), the two washes (W1 and W2), and the SDS eluate (E), 4 μl each, were subjected to immunoblotting to determine their hRad54 content.

DNA supercoiling and DNA strand opening by hRad54 - Purified hRad54 exhibits DNA-dependent ATPase and DNA supercoiling activities (Swagemakers et al, 1998; Tan et al, 1999; Ristic et al, 2001). Tan et al (1999) showed an ability of hRad54 to alter the DNA linking number of a nicked plasmid in the presence of DNA ligase in a hRad54 ATP dependent manner. As indicated in the schematic in Figure 2 that depicts the basis for tracking-induced DNA supercoiling by hRad54, scanning force microscopy (SFM) was utilized to provide evidence that hRad54 tracks on DNA when ATP is hydrolyzed (Ristic et al, 2001).

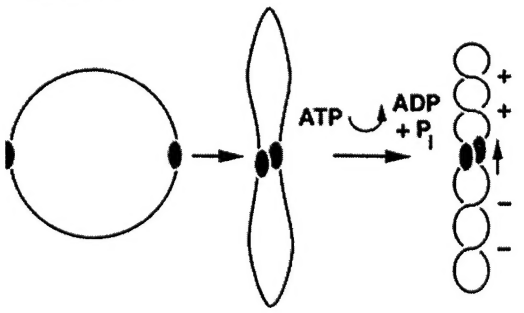
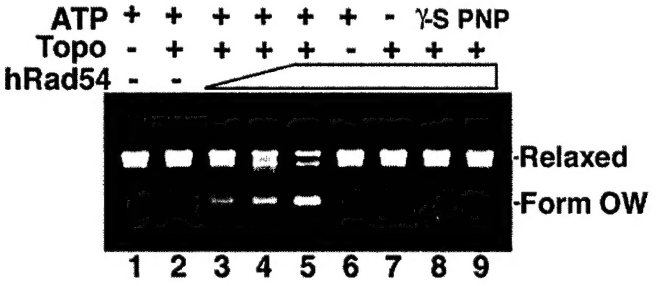


Figure 2. Basis for hRad54 induced supercoiling. The free energy from ATP hydrolysis fuels the tracking of a hRad54 oligomer on DNA, producing a positively supercoiled domain ahead of protein movement and a negatively supercoiled domain behind (Ristic et al, 2001; Van Komen et al, 2000).

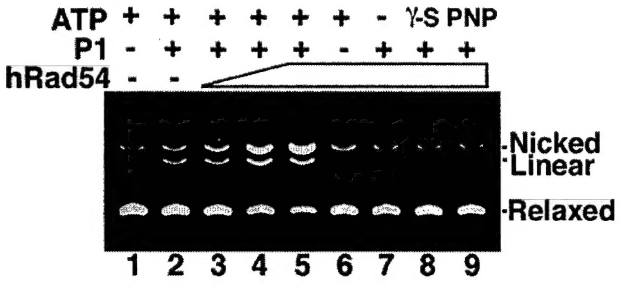
We have previously shown that the yeast Rad54 protein also tracks on DNA and, as a result, generates positive and negative supercoils in the DNA substrate (Van Komen et al, 2000). Removal of the negative supercoils by treatment with *E. coli* topoisomerase I leads to the accumulation of positive supercoils and the formation of an overwound DNA species called Form OW (Van Komen et al, 2000). Here we used the same strategy to examine the ability of hRad54 to supercoil DNA. As shown in Figure 3, in the presence of topoisomerase, purified hRad54 protein readily induces a linking number change in the DNA (Van Komen et al, 2000). A dependence on ATP hydrolysis in promoting a linking number change is evident as Form OW fails to form when omission or substitution of ATP with a non-hydrolyzable analogue (ATP-γ-S or AMP-PNP) occurs (Figure 3).

Figure 3. hRad54 supercoils DNA. Increasing amounts of hRad54 (200, 400, and 750 nM in lanes 3 to 5, respectively) was incubated with topologically relaxed DNA (20 μM nucleotides) and *E. coli* topoisomerase I in buffer that contained ATP. The highest amount of hRad54 (750 nM) was also incubated with the DNA substrate in the absence of topoisomerase (lane 6) and in the presence of topoisomerase but with the omission of ATP (lane 7) or the substitution of ATP by ATP-γ-S (γ-S; lane 8) and AMP-PNP (PNP; lane 9). DNA alone (lane 1) or DNA incubated with topoisomerase (lane 2) were also included. The reaction mixtures were run in a 1% agarose gel, which was treated with ethidium bromide to reveal the DNA species.



Next, we addressed whether the negative supercoils generated as a result of hRad54 tracking on the DNA substrate (Ristic et al, 2001; Figure 2 and 3) leads to transient DNA strand opening, by examining the sensitivity of a relaxed DNA template to the single-strand specific nuclease P1, as we have previously done for yeast Rad54 (Van Komen et al, 2000). As indicated by the accumulation of nicked circular and linear DNA forms, incubation of topologically relaxed DNA with hRad54 rendered the relaxed DNA substrate sensitive to P1 nuclease (Figure 4). Furthermore, like the DNA supercoiling reactions, the DNA strand opening reaction is also completely dependent on ATP hydrolysis (Figure 4).

Figure 4. hRad54 promotes DNA strand opening. Increasing amounts of hRad54 (200, 400, and 750 nM in lanes 3 to 5, respectively) was incubated with topologically relaxed DNA (20 μM nucleotides) and P1 nuclease in buffer that contained ATP. The highest amount of hRad54 (750 nM) was also incubated with the DNA substrate in the absence of P1 (lane 6) and in the presence of P1 but with the omission of ATP (lane 7) or the substitution of ATP by ATP-γ-S (γ-S; lane 8) and AMP-PNP (PNP; lane 9). DNA alone (lane 1) and DNA incubated with P1 in the absence of hRad54 (lane 2) were also included. The reaction mixtures were run in a 1% agarose gel containing 10 μM ethidium bromide.



Activities of hRad54 are stimulated by hRad51 – The results presented here (Figure 1) and elsewhere (Golub et al, 1997) have established a specific interaction between hRad51 and hRad54 suggesting a possible enhancement of the biological activities of these proteins. To test this, we first examined if the hRad54 ATPase activity would be enhanced by interaction with hRad51. As shown in Figure 5, when hRad54 was combined with hRad51 a much higher rate of ATP hydrolysis was seen. The fact that yRad51 was ineffective in this reaction (Figure 5) indicates that the action of hRad51 is specific. Even though hRad51 is known to have a weak ATPase activity (Benson et al, 1994), the fact that the hrad51 K133R mutant protein, which binds but does not hydrolyze ATP (Morrison et al, 1999), was just as effective in promoting ATP hydrolysis (data not shown) strongly indicated that the increase in ATP hydrolysis was due to enhancement of the hRad54 ATPase function.

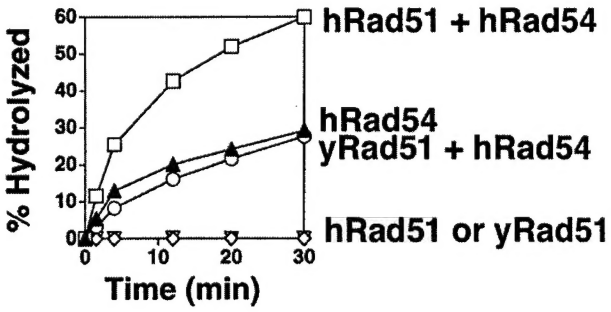


Figure 5. hRad54 ATPase activity is stimulated by hRad51. - hRad54 was incubated with ϕX replicative form I DNA (30 μM nucleotides) and 1.5 mM [γ-^{32}P] ATP for the indicated times, and the level of ATP hydrolysis was quantified by thin layer chromatography. ATPase activity was also measured for hRad51 alone, yRad51 alone, and the combinations of hRad54/hRad51 and hRad54/yRad51. The protein concentrations were: hRad54, 60 nM; hRad51, 400 nM; yRad51, 400 nM. In every case, negligible ATP hydrolysis was seen when DNA was omitted (data not shown).

We next asked whether the DNA supercoiling activity of hRad54 would also be upregulated by hRad51. The results showed that hRad51 stimulates the supercoiling reaction, as indicated by a much higher level of Form OW DNA (Figure 6A). Since negative supercoiling generated by hRad54 leads to DNA strand opening (Figure 4), we thought that hRad51 may also promote this activity. Indeed, the inclusion of hRad51 greatly elevated the nicking of the relaxed DNA substrate by P1 nuclease (Figure 6B). Even with the inclusion of hRad51, no Form OW DNA or nicking of DNA was seen when ATP was omitted or substituted by the non-hydrolyzable analogues ATP-γ-S and AMP-PNP (Figure 6 A & B). Thus, the results revealed that hRad51 markedly stimulates the ability of Rad54 to supercoil DNA and unwind DNA strands. The hrad51 K133R protein was just as effective as wild type hRad51 in enhancing the DNA supercoiling and strand opening activities of hRad54 (data not shown). Furthermore, we found that yRad51 does not stimulate the hRad54 activities (data not shown), thus indicating a high degree of specificity in the hRad51 action.

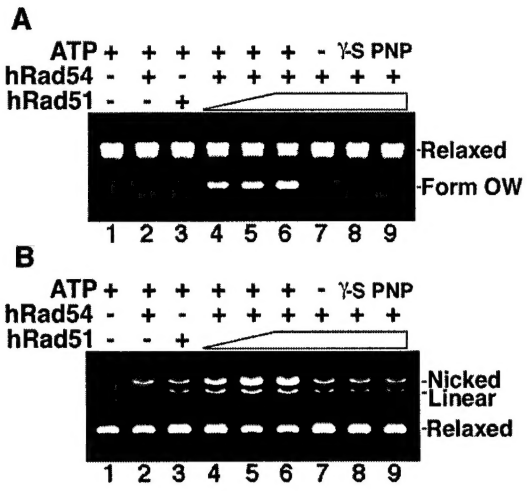


Figure 6. hRad51 stimulates hRad54 supercoiling and strand opening activities. *(A)* Relaxed DNA (20 μM nucleotides) was incubated with hRad54 (75 nM in lanes 2, and 4 to 9) and hRad51 (80, 160, and 240 nM in lanes 4 to 6, respectively) in the presence of ATP and *E. coli* topoisomerase I. The highest amount of hRad51 (240 nM) was incubated with substrate and topoisomerase I but without hRad54 (lane 3) and also with hRad54 (75 nM) but with the omission of ATP (lane 7) or its substitution with ATP-γ-S (γ-S; lane 8) or AMP-PNP (PNP; lane 9). DNA alone was analyzed in lane 1. After deproteinization, the reaction mixtures were run in a 1% agarose gel, which was treated with ethidium bromide to stain the DNA species. ***(B)*** Relaxed DNA was incubated with hRad54 (75 nM in lanes 2 and 4 to 9) and hRad51 (80, 160, and 240 nM in lanes 4 to 6, respectively) in the presence of ATP and P1 nuclease. The highest amount of hRad51 (240 nM) was incubated with substrate and P1 but without hRad54 (lane 3) and also with hRad54 (75 nM) but with the omission of ATP (lane 7) or its substitution with ATP-γ-S (γ-S; lane 8) or AMP-PNP (PNP; lane 9). DNA alone was run in lane 1. Analysis was carried out in a 1% agarose gel that contained 10 μM ethidium bromide.

hRad51 and hRad54 co-operate in homologous DNA pairing - The RecA/Rad51 class of general recombinases is central to recombination processes by virtue of their ability to catalyze the homologous DNA pairing reaction that yields heteroduplex DNA joints (Bianco et al, 1998; Sung, 2000). Since hRad51 and hRad54 physically interact (Golub et al, 1997; Figure 1), and hRad51 enhances the various activities of hRad54 (Figure 5 and 6), it was of considerable interest to examine the influence of hRad54 on hRad51-mediated homologous DNA pairing.

The homologous pairing assay monitors the incorporation of a ^{32}P-labeled single-stranded oligonucleotide into a homologous supercoiled target (pBluescript) to give a D-loop structure (Figure 7A). As reported before (Mazin et al, 2000a) and reiterated here (Figure 7B), hRad51 by itself is not particularly adept at forming D-loop. Importantly, inclusion of hRad54 rendered D-loop formation possible. D-loop formation by the combination of hRad51 and hRad54 requires ATP hydrolysis, as no D-loop was seen when ATP was omitted or when it was replaced by either ATP-γ-S or AMP-PNP (Figure 7B). Significantly, time course analysis revealed a cycle of rapid formation and disruption of D-loop, such that the D-loop level reached its maximum by 1 min but declined rapidly thereafter (Figure 7, B and D). In fact, by the reaction endpoint of 6 min, little or no D-loop remained (Figure 7, B and D). Such a cycle of D-loop synthesis and reversal has also been observed for *E. coli* RecA (Shibata et al, 1982). Since the RecA-ssDNA nucleoprotein filament disassembles upon ATP hydrolysis (Bianco et al, 1998), we considered the possibility that the dissociation of D-loop seen here (Figure 7B) could be related to ATP hydrolysis-mediated turnover of hRad51. To test this premise, we used the hrad51 K133R mutant protein, which binds but does not hydrolyze ATP (Morrison et al, 1999), with hRad54 in the D-loop assay. True to prediction, with hrad51 K133R, the D-loop amount increased with time, reaching a much higher final level than when hRad51 was used (Figure 7, B, C and D); by 5 min, 23% of the input ssDNA, or 55% of the pBluescript plasmid DNA, had been incorporated into the D-loop structure. As expected, with both hRad51/hRad54 and hrad51 K133R/hRad54, formation of D-loop required both the 90-mer substrate and pBluescript target, and substitution of the pBluescript DNA with the heterologous ϕX174 DNA completely abolished D-loop formation (data not shown).

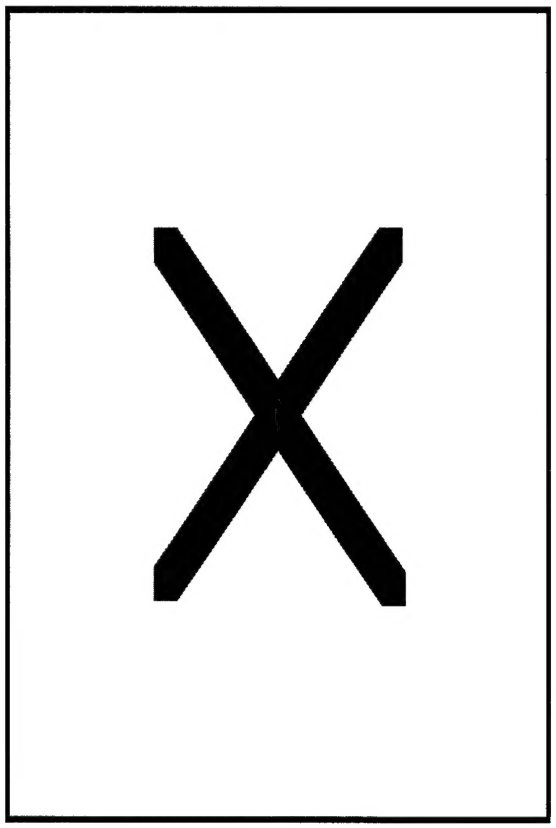


Figure 7. D-loop formation by hRad51 and hRad54. ***(A)*** Schematic of assay. A radiolabeled 90-mer DNA pairs with a homologous duplex target to yield a D-loop. ***(B)*** hRad51 alone (lanes 2 and 3), hRad54 alone (lanes 4 and 5), and the combination of hRad51 and hRad54 (lanes 6 to 14) were incubated at 30°C for the indicated times with the DNA substrates in the presence of ATP (lanes 2 to 11), ATP-γ-S (γS; lane 13), AMP-PNP (PNP; lane 14), or in the absence of nucleotide (lane 12). In lane 1, the DNA substrates were incubated in buffer without protein. The protein and DNA concentrations were: hRad51, 800 nM; hRad54, 120 nM; 90-mer oligonucleotide, 2.5 μM nucleotides or 27.7 nM oligonucleotide; pBluescript supercoiled DNA, 35 μM base pairs or 11.6 nM of plasmid. ***(C)*** The homologous pairing activity of hrad51 K133R was examined with hRad54 as described for hRad51 above. ***(D)*** The results in lanes 6 to 11 of B (open circles) and C (filled circles) were graphed.

APPENDED INFORMATION

(1) KEY RESEARCH ACCOMPLISHMENTS TO DATE:

- **Demonstrate hRad51 and hRad54 directly interact using purified proteins.**
- **Demonstrate that in the presence of ATP hRad54 supercoils DNA and promotes a transient DNA strand opening.**
- **Demonstrate that hRad51 stimulates hRad54 ATPase, DNA supercoiling and transient DNA strand opening activities in the presence of ATP.**
- **Demonstrate that hRad54 works in concert with hRad51 to promote D-loop formation.**

(2) REPORTABLE OUTCOMES

Presentations:

- Invited workshop speaker and poster presentation, Keystone Research Symposia on Molecular Mechanisms of DNA Replication and Recombination, Snowbird, UT. Title of Poster and Presentation "Functional Interactions between Rad51 and Rad54 in Heteroduplex Joint Formation".

- Presentation at the Molecular Medicine Annual Retreat, Department of Molecular Medicine University of Texas Health Science Center at San Antonio. Title of presentation "Functional Interactions Between Rad51 and Rad54 Provide Efficient Homologous DNA Pairing and Strand-Exchange".

- Abstract sent for a poster presentation at the Era of Hope Department of Defense Breast Cancer Research Program Meeting, Orlando Florida, September 2002. Title of presentation "Rad51 and Rad54 Cooperate in Efficient Heteroduplex DNA Joint Formation".

Publications:

Van Komen, S., Petukhova, G, Sigurdsson, S. and Sung P. Functional Crosstalk Among Rad51, Rad54, and RPA Heteroduplex DNA Joint Formation. manuscript submitted.

Sigurdsson S., Guo X.Z., Van Komen, S., Petukhova, G. and Sung, P. Homologous DNA Pairing by Human recombination Factors Rad51 and Rad54. manuscript submitted.

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